Investigation of the Physiological Responses of Belugas to "Stressors" to Aid in Assessing the Impact of Environmental and Anthropogenic Challenges on Health

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Award Number: N00014-11-1-0437
http://searesearch.org

LONG-TERM GOALS

The overall top level goal of this effort is to investigate the physiological i.e. neuroimmunoendocrinological responses of beluga whales to "stressors". "Stressor events" will allow for a better understanding and characterization of the relationships among hormones (e.g. cortisol, corticosterone, adrenocorticotropin hormone, aldosterone, catecholamines) in different matrices (blood, saliva, blow, feces) in conjunction with immune function. In addition, "stressor events" will enable us to define and compare the quantitative and temporal relationships of hormones across the different matrices.

OBJECTIVES

The objectives of this effort are: 1) To monitor the neuroimmunoendocrinological responses (via saliva and blood) of three resident aquarium belugas before and after the introduction and throughout the adaptation process of seven new belugas to their habitat and to measure the neuroimmunoendocrinological responses of 5 belugas to transport and 2) To monitor the

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
30 SEP 2011		**00-00-2011 to 00-00-2011**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Investigation of the Physiological Responses of Belugas to 'Stressors' to Aid in Assessing the Impact of Environmental and Anthropogenic Challenges on Health	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Mystic Aquarium, a division of Sea Research Foundation,55 Coogan Blvd,Mystic,CT,06355**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**	**Same as Report (SAR)**	**7**	

neuroimmunoendocrinological responses (via blood, saliva, blow and feces) of 2-3 aquarium resident whales before and after the occurrence of a known stressor.

APPROACH

Seven belugas, *Delphinapterus leucas* (one male 22 years of age, four females 9-26 years of age, two calves- 1 male and 1 female < 2yrs) were transported from Shedd Aquarium, Chicago, IL to Mystic Aquarium, Mystic, CT in the fall of 2008 and remained at Mystic Aquarium until the spring of 2009 while exhibit modifications occurred at the Shedd Aquarium. The transported belugas were initially placed into a holding pool physically separated from the three resident belugas (one male and two females approximately 27 yrs) at Mystic Aquarium. However, all belugas could establish visual and auditory contact with each other. Blood and saliva samples had already been collected and archived for a subset of the transported belugas at time points before, during and after the transport and introduction. Catecholamines will be measured in blood via High Performance Liquid Chromatography; adrenocorticocotropin hormone, cortisol and aldosterone will be measured via established chemiluminescent and radioimmunoassays at the Animal Health Diagnostic Center, Endocrinology, College of Veterinary Medicine or via enzyme immunoassay (EIA) at the Mystic Aquarium; immune function including the ability of lymphocytes to proliferate and quantification of lymphocyte subsets (via flow cytometry) will be assessed. Methodology for quantification of cortisol in beluga saliva will be worked out as well as determination of diurnal patterns if any in saliva. The relationships among hormones before after the stressors will be evaluated as well as the relationships between the hormones and immune function.

To fulfill objective 2, samples of blood, saliva, blow and feces will be collected at time points before, during and after out of water examinations for 2-3 aquarium resident whales. Catecholamines will be measured in blood via High Performance Liquid Chromatography; adrenocorticocotropin hormone, cortisol and aldosterone will be measured via established chemiluminescent and radioimmunoassays at the Animal Health Diagnostic Center, Endocrinology, College of Veterinary Medicine or for cortisol via EIA at the Mystic Aquarium; immune function including the ability of lymphocytes to proliferate, quantification of lymphocyte subsets and phagocytosis and respiratory burst of neutrophils and monocytes will be assessed via flow cytometry. Methodology for quantification of cortisol in beluga saliva and blow and corticosteroids in feces will be developed. The relationships among hormones before and after the stressors will be evaluated as well as the relationships between the hormones and immune function. The quantitative and temporal relationships of corticosteroid hormones across the different matrices will be evaluated.

Tracy Romano (P.I.) is primarily responsible for overseeing all sample collection and analyses, the development and transition of hormonal assays, and data integration and analyses. She will coordinate the project with the Co-Investigators both (on-site and off-site), she will write the results of the research in manuscript format for publication and present the research at scientific meetings and public forums.

Tracey Spoon (Co-P.I.) will play an activate role in sample collection, assay development and coordination of sample analyses in the laboratory, and work closely with the technician and graduate student on the analyses in the laboratory. She will work with the P.I. on data integration and analysis as well as publication.

Steve Lamb (Co-P.I.) will be responsible for the ACTH, aldosterone and cortisol assays that will be conducted at the AHDC at Cornell and advise and assist the P.I. and Co-P.I. with hormonal assay development and validation and transition of technology to Mystic Aquarium.

Laura Thompson, PhD student at the University of Connecticut (UCONN), Department of Marine Biosciences will contribute to development of an assay for measuring stress hormones in cetacean blow as well as play a role in carrying out the immune function assays in the laboratory and assist with sample collection and data analyses.

WORK COMPLETED

Much progress has been made on the archived samples from objective one in which belugas were sampled before, during and after transport and before, during and after novel introductions. Specifically, samples have been assayed for the catecholamines (epinephrine, norepinephrine and dopamine) via High Peformance Liquid Chromatography with Electrochemical detection; cortisol, ACTH and aldosterone using established chemiluminescent and radioimmunoassays at the Animal Health Diagnostic Center, Endocrinology Laboratory at the College of Veterinary Medicine, Cornell University; quantification of phagocytosis and respiratory burst of neutrophils and monocytes (carried out on fresh samples); complete blood cell counts (carried out on fresh samples) and serum chemistries. Moreover, a subset of experiments were carried out to determine if the same end results would be obtained if utilizing serum vs. Na heparin plasma vs. EDTA plasma for cortisol, adosterone and ACTH.

In regards to objective 2, one out of water event for 3 whales has been conducted. Preliminarily, whales were trained or behaviors reinforced for blood, blow, saliva and feces collection under behavioral control. Blood, blow, saliva and feces were taken at the same time points before, during and after the out of water event (stretchered, removed from the water and placed on the deck for physical examination (approximately 30 minutes). Blood samples that needed to be run right away included phagocytosis and respiratory burst of neutrophils and monocytes and complete blood cell counts. The others were archived for subsequent analysis of catcholamines, cortisol, ACTH, aldosterone, lymphocyte proliferation and immunophenotyping. Saliva, blow and feces were collected and archived. Prior to the out of water event different collection methods were investigated for the saliva (100% cotton gauze (CG) vs. a viscose-polyestser gauze (VPG) vs. Salimetrics™ oral swabs (SOS)) and blow (number of exhales; nylon stocking vs. cotton gauze vs. nitex membrane vs. tulle as an absorbent; 50 ml tubes vs. 250 ml Nalgene bottle vs. petri dish). Preliminary analyses have been conducted to examine the efficacy of using a commercially-available human serum cortisol EIA kit (Cayman Chemical Company, Ann Arbor, MI) to measure cortisol levels in beluga saliva and blow.

RESULTS

The results obtained thus far for objective one including catecholamine and cortisol analyses and phagocytosis and respiratory burst of neutrophils and monocytes have been accepted for publication (Spoon and Romano, *In Press*). Briefly, mean plasma concentrations of epinephrine and norepinephrine for resident belugas showed significant differences among baseline, arrival and acclimation samples in regards to the introduction of the new belugas to their habitat. All three subjects showed increases in epinephrine, norepinephrine and dopamine between baseline and arrival and a subsequent decrease between arrival and acclimation. However, they did not show significant differences in serum cortisol or ACTH levels among baseline, arrival, and acclimation. Hematological

analysis revealed only significant differences in lymphocytes with a decrease upon introduction of the other whales compared to baseline. Resident belugas exhibited a concomitant decrease in neutrophil and monocyte phagocytosis associated with the introduction of the tranported belugas.

All four transported belugas exhibited an increase in epinephrine and norepinephrine between baseline and arrival samples and a decrease between arrival and acclimation samples. Mean plasma dopamine levels did not show significant differences among the three sampling periods. Compared to the resident belugas, transported belugas displayed significant differences in cortisol and ACTH levels among baseline, arrival and acclimation samples, with all four transported belugas displaying an increase in cortisol and ACTH between baseline and arrival samples and a decrease between arrival and acclimation samples. Hematological analysis revealed a significant change in lymphocytes between baseline and arrival and acclimation. Transported belugas exhibited an attendant increase in phagocytois and respiratory burst activity immediately following transport.

The difference between the resident and transported belugas suggest that the transport process represented a more potent stressor that the changes in environment experienced by the resident belugas. The results indicate that both scenarios represent model systems that can be used to examine the stress reponse in cetaceans.

Cortisol concentration measured in serum showed a strong positive correlation with cortisol measurements in matched samples of sodium heparin plasma or EDTA plasma. ACTH measured in EDTA plasma showed a strong positive correlation with ACTH measured in matched sodium heparin plasma but a much weaker relationship to cortisol measured in matched serum samples. These results indicate that EDTA plasma, sodium heparin plasma, and serum provide very similar measures of cortisol in beluga blood and that EDTA and sodium heparin but not serum provide very similar measures of ACTH in beluga blood. These results allow for a consolidation of blood tubes and therefor a decrease in number for carrying out neuroimmunoendocrinological assessment before, during and after stressors.

For the first out of water event, the 3 whales showed different responses. In regards to blood cells, female whale one showed increasing numbers of white blood cells and neutrophils from baseline after being out of the water 10 and 20 minutes and began to show a decrease after 30 min, while female 2 remained fairly constant until the post evaluation 24 hrs later showing an increase in wbc, neutrophils and lymphocytes. The male remained relatively constant in wbc and neutrophils with a peak level after 20 min but showed a decrease in lymphocytes in the post samples 1 hr and 24 hrs later. Female one showed a decrease in neutrophil and monocyte phagocytosis and respiratory burst after 30 min on the beach and post 60 min. Values began to rise 24 hrs later and increased from baseline in the following post samples. Female two showed a decrease in phagocytosis and respiratory burst for neutrophils and monocytes after 30 min on the beach, while the male beluga increased in these measures.

Prelimary analyses have been conducted to examine the efficacy of using a commercially-available human serum cortisol EIA kit (Cayman Chemical Co., Ann Arbor, MI) to measure cortisol in beluga saliva. To test for the presence of factors that may interfere with the assay and thus indicate a need for extraction, saliva samples from all three collection materials (CG, VPG, SOS) were serially diluted and compared for parallelism with the standard curve. The slopes of the dilution curves for the CG, VPG and SOS showed no significant differences from that of the standard curve indicating that the cortisol extraction from beluga saliva is not necessary. The slopes for SOS and CG dilution curves

were closer to 1.0, the ideal slope, than that of VPG. The SOS and VPG exhibited similar mean cortisol levels although the VPG series had a substantially greater standard deviation. In contrast, the observed cortisol concentration was notably lower in the CG samples that the matched SOS samples and the standard deviation was higher. The cortisol concentration from the SOS was similar to other baseline samples collected using SOS and VPG while the CG sample appeared abnormally low. Examining visual representations of the dilution points for SOS, VPG, CG and standard curve compared to an ideal line (slope = 1), SOS results are most similar to the standard curve in the degree of deviation from ideal (Figure 1a,b). Based on the comparison of the slopes and the notably lower standard deviation, SOS appear to the best option for salivary cortisol analyses.

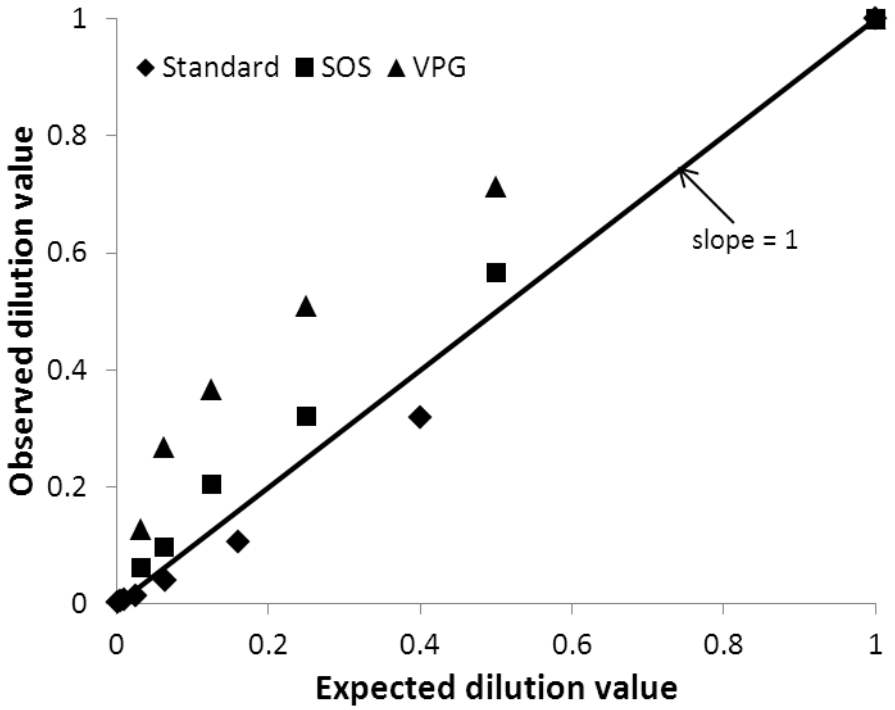

Figure 1a. Correlation between normalized expected and observed cortisol concentrations in serially diluted saliva samples collected using SOS and VPG compared with the standard cortisol curve.

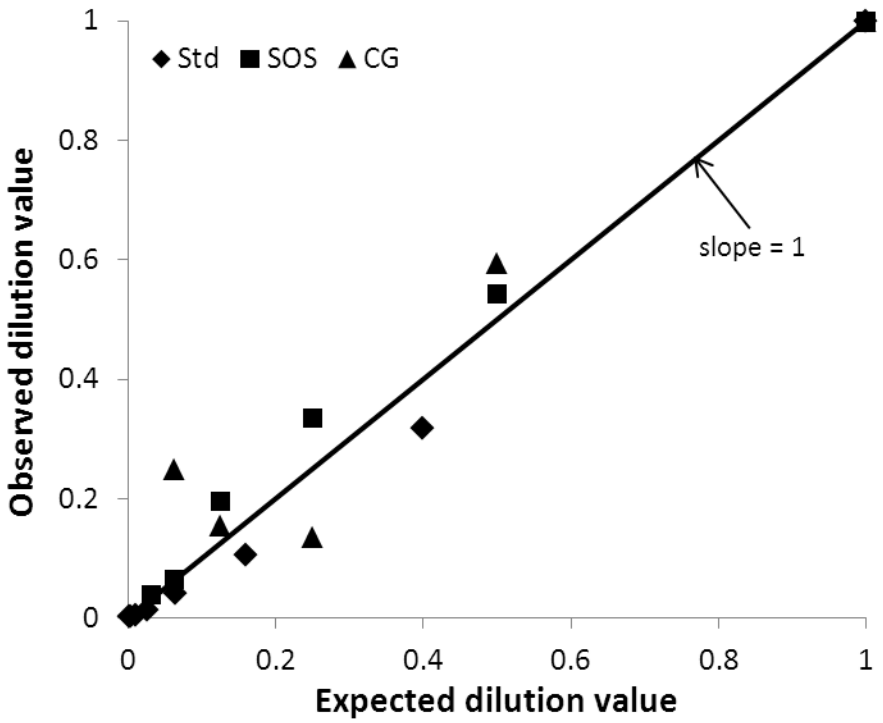

Figure 1b. Correlation between normalized expected and observed cortisol concentrations in serially diluted saliva samples collected using SOS and CG compared with the standard cortisol curve.

Regarding the blow, we determined that the utilization of collection material secured over a petri dish and inverted directly over the blow hole is a better collection method that the use of inverted conical tubes (50 ml) or nalgene bottles (250ml) containing collection material based on volume recovered. Among the collection materials used, tulle and cotton gauze resulted in the recovery of the smallest volumes on average (<40 µl); while nylon stocking and nitex membrane resulted in recovery of sample volumes averaging >50 µl. The nylon stocking appears to absorb a larger volume of blow than the nitex membrane (4 exhales; 134.8 µl and 80.9 µl; 8 exhales 167.5 µl and 159.0 µl). Serial dilution tests were run using samples collected with nylon stocking and nitex membrane to determine interfering substances. The nitex membrane displayed less interference in the cortisol assay and thus was chosen for future collection of blow in belugas.

IMPACT/APPLICATIONS

There is increasing concern regarding the potential effects of anthropogenic sound on marine mammals. The U.S. Navy is under continuous scrutiny with regards to sonar exercises and impacts on marine mammals. While studies have been conducted on behavioral and auditory responses in marine mammals with respect to anthropogenic sound there is a lack of scientific data and knowledge of the physiological impacts of loud sound exposure on marine mammals. There are many limitations and constraints in investigating the effects of anthropogenic sound as a "stressor" and impacts on the physiology of marine mammals. Despite these limitations there is a recognized need for such studies.

Investigation of the physiological response to stressors is very difficult in marine mammals given the difficulty in imposing stressors on marine mammals that will elicit a response, the lack of validated assays for measuring stress hormones, the difficulty in obtaining samples without causing stress, and obtaining a large enough sample size to draw significant conclusions. We are uniquely positioned at Mystic Aquarium, a division of Sea Research Foundation, Inc. to overcome the above obstacles and can provide a better understanding of the relationships among hormones in different matrices and in relation to immune function after stressor events. We can also define and compare the quantitative and temporal relationships of hormones across different matrices. This basic information is needed to lay the groundwork for understanding the impact of anthropogenic sound on marine mammals individually and at the population level.

RELATED PROJECTS

Title: Variability of Hormonal Stress Markers Collected from a Managed Dolphin Population

PI: Dorian Houser, PhD National Marine Mammal Foundation

Longitudinal study of a large dolphin population to characterize stress markers in different matrices. Proposed effort also includes investigating the responsiveness of the thyroid and corticosteroid hormone production pathways.

Title: Pathophysiology of Stress in Wild and Managed-Care Bottlenose Dolphins (*Tursiops truncatus*)
PI: Pat Fair, PhD National Ocean Service

Study investigating hormones and immune function in wild dolphins vs. two different populations of managed-care dolphins (Aquarium setting vs. San Diego Bay).

PUBLICATIONS

Spoon, T.R. and T.A. Romano. Neuroimmunological Response of Beluga Whales (*Delphinapterus leucas*) to Translocation and a Novel Social Environment. Brain, Behavior and Immunity [In Press]